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Research Article

A Novel Approach to Assess the Dynamics of Extra-Chromosomal Circular Ribosomal DNA in Human Cells

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Abstract

Several nutrient-signaling pathways that extend life span have been described in model organisms. Thus, parallel and redundant signaling pathways that are similar across species might be subject to experimental manipulation. Here, we develop a PCR-based technique for testing the hypothesis that mitotic accumulation of extra-chromosomal ribosomal DNA circles might also determine life span in human cells. Using resveratrol, a phytochemical that counters age-related signs, we find treatment-dependent subcellular accumulations of extra-chromosomal 5S ribosomal DNA in human cell lines. These data suggest an association between DNA circles and intrinsic aging and demonstrate the utility of a PCR-based technique for studying the accumulation of dysfunctional molecules that promote senescence.

Keywords: Extra-chromosomal circular DNA; Aging; Genome stability; Mobile DNA; Homologous recombination; Ribosomal DNA; DNA circles; Resveratrol; SIRT1

Abbreviations: EccDNA: Extrachromosomal circular DNA; EccrDNA: Extrachromosomal circular ribosomal DNA; ERV: Endogenous Retroviruses; rDNA: Ribosomal DNA; LINE: Long Interspersed Nuclear Elements; SINE: Short Interspersed Nuclear Elements; SIRT1: Silent Information Regulator T1

Introduction

Aging appears to be plastic and can be manipulated by genetic and nutritional intervention. For example, activation of the silent information regulator T1 (SIRT1) pathway increases the life span of model organisms such as yeast and mice. SIRT1 is a NAD⁺-dependent deacetylase that directly links transcriptional

regulation to intracellular metabolism [1]. Among the signaling cues that activate SIRT1 pathways, is the polyphenol molecule resveratrol. SIRT1 activation

by resveratrol triggers a broad range of transcription factors and co-regulators that mediate key mechanisms in the cell cycle, cell growth and apoptotic and autophagic programs of cell death [2-4]. Other intracellular mechanisms activated by SIRT1-dependent

pathways are those related to DNA stability and DNA replication. For example, using the budding yeast (*Saccharomyces cerevisiae*) as a model for elucidating signaling pathways that control life span, Sinclair and Guarente [5] showed, that increased activity of SIR2

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(the yeast ortholog of mammalian SIRT1) suppresses the gradual accumulation of extra-chromosomal circular DNA (eccDNA). As its name implies, eccDNA are circular molecules propagated extra-chromosomally from repetitive and non-repetitive genomic regions of various species including, humans [6-9].

As in yeast, human ribosomal DNA (rDNA) is also organized in tandem rDNA repeats with five 43 kb rDNA (*rna45s5*) clusters encoding a large 45S rRNA precursor that is post-transcriptionally processed into 28S, 18S and 5.8S rRNAs [10,11]. 5S rRNA is encoded by a separate 5S rDNA (*rn5s1*) cluster with 100-150 copies of a 2.2kb rDNA tandem repeat [12,13]. Investigations into the genomic architecture of human rDNA clusters reveal significant meiotic re-arrangement of about 11% per generation per gene cluster [14], with 5SrDNA molecules undergoing significant replicative steps [7]. In humans, SIRT1 expression is also associated with rDNA re-arrangements suggesting a conserved composition and function of anti-aging pathways. However, there is little experimental evidence for these association pathways, in particular whether activation of SIRT1 or reduction of eccrDNA could be considered for the prevention of specific diseases. In addition, there is no sensitive platform for functional screening of human eccrDNA which allows for the precise and accurate analysis underlying the formation of eccrDNA molecules. To address this technical limitation, we developed and validated a PCR-based protocol in combination with a nuclear transport assay for the quantitative analysis of eccrDNA molecules. To further support its practical use, we tested whether this technique could identify the occurrence of additional eccDNA species in human cell lines.

Materials and Methods

Cell culture, drug treatment and collection of human cells

The following adherent human epithelial cell lines were propagated under standard culture conditions (37°C, 5% CO₂) and as recommended by the manufacturer (ATCC, Manassas, VA, USA): HEK-293 (embryonic kidney, CRL-1673); SH-SY5Y (neuroblastoma, CRL-2266) and; MCF7 (mammary adenocarcinoma, HTB-22). Resveratrol treatment (50 µM, final concentra-

tion, 6 h/48 h), was initiated at a cellular confluence level of about 70%. For nuclear transport studies, the lectin wheat germ agglutinin (WGA) was used to block nuclear transport (0.1 mg/ml, final concentration, 12 h) and the lectin, concavalin A (ConA), as control (0.1 mg/ml, final concentration, 12 h). At the end of a given treatment period, cells were washed with PBS, scraped off their dishes and collected for further analysis or stored (-80°C).

Resveratrol-dependent changes in gene expression using quantitative PCR (QPCR)

Following resveratrol treatment (48h), HEK-293, MCF7 and SH-SY5Y cells were collected and total RNA was prepared with the RNeasy RNA-isolation system/Qia shredder according to the manufacturer's specifications (Qiagen, Valencia, CA, USA). Following the determination RNA concentrations and integrity, complementary cDNA was generated with the Superscript III First Strand Synthesis System for RT PCR (Invitrogen, Carlsbad, CA, USA). QPCR was conducted on a Mastercycler ep gradient S (Eppendorf AG, Hamburg, Germany) using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total sample volume of 20 µl. Gene-specific DNA primers were either used as previously reported [15] or designed using the integrated DNA Technologies Primer Quest tool (IDT, Coralville, IA, USA). Expression was accessed for *sirt1*, *rn5s1* (5SrRNA) and *rna45s5* (45S pre-ribosomal RNA) using the following primers: *β-actin* forward: 5'-CAG CCA TGT ACG TTG CTA TCC AGG-3'; *β-actin* reverse: 5'-AGG TCC AGA CGC AGG ATG GCA TG-3'; *rn5s1* forward: 5'-GAT CTC GTC TGA TCT CGG AAG CTA AG-3'; *rn5s1* reverse: 5'-AAA GCC TAC AGC ACC CGG TAT T-3'; *rna45s5* forward: 5'-GAA CGG TGG TGT GTC GTT C-3' *rna45s5* reverse: 5'-GCG TCT CGT CTC GTC TCA CT-3'; *sirt1* forward: 5'-CTG TAG ACT TCC CAG ATC TTC CAG-3'; *sirt1* reverse: 5'-GTG ACA GAG AGA TGG CTG GAA TTG-3'.

Extraction of total eccDNA and relative quantification of eccrDNA circles

Resveratrol treated HEK-293, MCF7 and SH-SY5Y cells and controls (DMSO) were separated into their nuclear and cytoplasmic fractions according to the

manufacturer's instructions (CellLytic Nuclear Extraction Kit, Sigma, St. Louis, MO, USA). Both fractions were exposed to standard Na-acetate/ethanol precipitation and sensibly re-suspended in 100 µl TE buffer. To eliminate chromosomal DNA and linear fragments, individual samples were purified with the MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA). Then, samples were sequentially treated with exonuclease III (20 U/µl, 1h, 37°C), RNase A (50 µg/ml, 30 min, 37°C) and proteinase K (100 µg/ml, 1h, 37°C) followed by another purification and final elution in 20 µl of a 10 mM Tris buffer, pH 8.5. QPCR was carried out as described above with the same DNA primer pairs for *m5s1* and *ma45s5* only this time measured against *sstI* satellite and *alu* repeats [*sstI* forward: 5'-GTG GTG GTG CAT GGC CCC C-3'; *sstI* reverse: 5'-GAG CTC CAG GAT CAC CAC AGC-3'; *alu* forward: 5'-GGC GGG CGG ATC ACG AGG TCA G-3'; *alu* reverse: 5'-CCC GGG TTC ATG CCA TTC TCC TG-3'.

PCR amplification eccDNA for Southern blot or DNA sequencing analysis

Mixed nuclear and cytoplasmic eccDNA was isolated and purified from HEK-293 cells as described above. To specifically linearize 5S rDNA circles (single BamHI restriction site), one half of each sample (10 µl) was treated with BamHI for 4 h, 37°C while the other half was sonicated 3 times with 10s pulses on wet ice (30% maximum power, 2 mm tip; Cell Disruptor, Heat-Systems Ultrasonics, Plainview, NY, USA) for linearization/fragmentation. Samples were purified and eluted in a final volume of 10 µl. To generate blunt ends, samples were treated with T4-DNA polymerase and dNTPs (Roche Applied Science, Indianapolis, IN, USA) for 20 min at 16°C and immediately purified. EcoRI adaptor oligonucleotides were ligated to the linearized and blunt-ended DNA pieces for 18h at 16°C and excess adaptors subsequently removed by gel filtration using Sephacryl S-400 spin columns according to the manufacturer's instructions (both: Universal Riboclone cDNA Synthesis System, Promega, Madison, WI, USA). Derivatives of eccDNA were then amplified using a high fidelity PCR system (Expand 20 kb plus PCR System, Roche Applied Science, Mannheim, Germany) and DNA primer matching the EcoRI adaptor sequence (forward and reverse primer: AAT

TCC GTT GCT GTC G; Promega, Madison, WI, USA). Reactions destined for DNA cloning and sequencing were exclusively recruited from the sonicated samples and subject to PCR elongation reactions at 4 min. Reactions used for Southern blot analysis were recruited from sonicated as well as BamHI restricted samples and subject to 15 min PCR elongation reactions.

Southern blot analysis of eccDNA

Linearized and amplified eccDNA samples were slowly spread on ethidium bromide-stained 0.8% agarose gels (20-30 mA). Then, gels were bathed in 0.25 M HCl for 15min followed by transfer solution (0.5 M NaOH, 1.5 M NaCl) for 30 min before being assembled for alkaline upward DNA transfer onto a BrightStar-Plus nylon membrane (Ambion/Life Technologies, Carlsbad, CA, USA) with transfer solution for 12-18 h. After a 5 min-bath in neutralizing solution (0.5 M Tris-CL, pH8.0), the membranes were dried and baked at 80°C between blotting paper for 3 h. Then, blots were pre-hybridized with UltraHyb solution (Ambion/Life Technologies, Carlsbad, CA, USA) at 42°C for 30 min and then hybridized for 12-16 h with a PCR-generated and gel-extracted DNA probe specific for rDNA (Strults et al., 2008) labeled using the BrightStar Psolaren-Biotin System (Ambion/Life Technologies, Carlsbad, CA, USA). Autoradiographs were scanned and images imported into Adobe Photoshop CS5.1 (Adobe Systems Incorporated, Mountain View, CA).

Identification and screening of eccDNA in HEK-293 cells

Linearized and amplified eccDNA samples were briefly separated on ethidium bromide-stained 1.5% agarose gels from which a fragment cluster ranging from approximately 100 bp – 4 kb was removed using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Purified DNA was ligated into the pGEM-T Easy vector system (Promega, Madison, WI, USA) and transformed into chemically competent *E. coli* DH5α (Invitrogen/Life Technologies, Carlsbad, CA, USA). Plasmid DNA from ampicillin-resistant and beta-galactosidase negative transformants (blue-white screening) was treated with EcoRI restriction endonuclease for 1 h at 37°C and analyzed via standard agarose gel electrophore-

sis (1.5%) to confirm presence of an insert. Only confirmed clones were selected for DNA sequencing using standard T7 sequencing primer (T7 RNA polymerase promoter). DNA sequences were screened for identity and genomic origin with the “Basic Local Alignment Search Tool” (BLAST; <http://www.ncbi.nlm.nih.gov/blast/>). To determine a potential driving-force for eccDNA mobilization, all confirmed sequences were analyzed with the repetitive sequence screening tool CENSOR [16]; (<http://www.girinst.org/censor/index.php>).

Results and Discussion

While yeast SIR2 has a stabilizing effect on the organism’s rDNA locus, the exact role of SIRT1 on human eccrDNA dynamics is unknown. Interestingly, SIRT1 associates with the human *rna45s5* locus which it transcriptionally regulates *via* the energy-sensing eNoSC protein complex [15,17]. To draw first conclusions about *rn5s1*, we determined the transcriptional

activity of *rn5s1* and *rna45s5*, as well as *sirt1*, in HEK-293 cells in response to resveratrol (non-significant trends in MCF7 & SH-SY5Y) (Figure 1A). Consistent with previous work [3,4], we found relevant transcriptional increases of *sirt1* itself (1.9-fold) but also *rna45s5* (1.8 fold) and *rn5s1* (3.5 fold) suggesting parallel regulation and presence of SIRT protein at both rDNA loci.

In gain-of-function mouse models of disease, over-expression of SIRT1 increases homologous recombination of the entire rodent genome [18]. This suggests that DNA stability/mobility depends, in part, on the activation of SIRT1- dependent protein complexes and signaling pathways, particularly in those cells involved in nutrient metabolism and cellular growth. Proper analysis and quantification of eccrDNAs is currently cumbersome - mainly due to their relatively low abundance in human cells and tissues [7]. We therefore sought to develop a sensitive QPCR-based approach with appropriate internal standards, suited for linearized eccrDNA

Figure 1: Resveratrol induces the expression of *sirt1*, and rDNA genes but does not alter *alu*-eccDNA in HEK-293 cells. **A)** QPCR-analysis reveals increases in relative gene expression for *sirt1* (1.9-fold), *rna45s5* (1.8-fold) and *rn5s1* (3.5-fold) with resveratrol (R) treatment (50 μ M in DMSO, 48h) compared to control (C) conditions (DMSO). **B)** At the same time, concentrations of *alu*-eccDNA in either nuclear or cytoplasmic compartment remained unaffected. The “relative *alu*-eccDNA index” compares *alu*-eccDNA with β -actin expression of the same treatment group *via* QPCR. Values are means $\pm$ SEM.

* indicates statistical significance of annotated P; n = 20 for all groups.

molecules. The *sstI* family for satellite repeats consists of 2.5 kb repeating units with approximately 400 copies within genomic clusters [19]. The most common *alu* repeats (1 million copies per haploid genome) are approximately 280 bp long and their average genomic separation of only about 3 kb increases the potential for genomic re-arrangement and eccDNA formation in mammalian cells. Such non-tandem repeats were previously detected in eccDNA pools from human cells [20,21]. Whereas *alu*-eccDNA was reliably detectable across nuclear and cytoplasmic compartments, *sstI*-eccDNA was detectable in most nuclei but rarely in cytoplasm (data not shown). To ensure integrity of the internal standard in response to resveratrol (50 μ M), cells were separated into their cytoplasmic and nuclear fractions and processed for isolation of eccDNA and RNA (reversely transcribed into cDNA). The quotient of *alu*-eccDNA and β -actin expression of the same cells (relative *alu*-eccDNA index) was independently derived for resveratrol-treated and untreated cells and com-

pared. Resveratrol treatment did not significantly affect the concentration of *alu*-eccDNA in either compartment (Figure 1B) supporting its suitability as internal standard.

Using our new technique, we tested if the modest resveratrol-dependent transcriptional changes in *m5s1* and *ma45s5* were reflected by nuclear and cytoplasmic patterns of the corresponding eccrDNAs (Figure 2A-C). Whereas the average cytoplasmic *m5s1*-eccrDNA concentration increased by 2.2-fold (HEK-293), 3.4-fold (MCF7) and 1.5-fold (SH-SY5Y), respectively, nuclear *m5s1*-eccrDNA was significantly decreased by about 60% (HEK-293), but unaffected in the other two cell lines. Changes in *ma45s5*-eccrDNA never reached statistical relevance (data not shown).

To confirm the presence of *m5s1*-eccrDNA in HEK-293 cells, we analyzed subcellular eccDNA extracts amplified with long-range PCR through Southern-blot analysis (Figure 2D) using a previously published and

Figure 2: Resveratrol significantly augments cytoplasmic *m5s1*-eccDNA in human cells. QPCR analysis (relative to *alu*-eccDNA) comparing resveratrol (R) treatment (50 μ M in DMSO, 48h) with control (C) conditions (DMSO) reveals significant increases of *m5s1*-eccDNA in the cytoplasm of **A.** HEK-293 cells (2.2-fold), **B.** MCF7 cells (3.4-fold) and **C.** SH-SY5Y cells (1.5-fold). Concomitantly, *m5s1*-eccDNA decreases by about 60% in HEK-293 cell nuclei but not in other cells. **D.** Southern blot, using a gene-specific labeled DNA probe, confirms *m5s1*eccDNA in HEK-293 cells (→). Cytoplasmic and nuclear eccDNA extracts are linearized/fragmented (sonication or BamHI), electrophoresed and blotted. Lower bands are consistent with 2.2kb *m5s1*eccDNA monomers. Only cytoplasmic samples display an additional signal ≥ 12 kb consistent with *m5s1*eccDNA multimers. QPCR-values are means $\pm$ SEM.

* indicates statistical significance of annotated P; n = 20 per group (**A-C**).

labeled *rn5s1*-eccrDNA probe for detection [7,14]. Initially in this process, all eccDNA molecules were linearized/fragmented with sonication or BamHI restriction (*rn5s1*-eccrDNA has a unique BamHI restriction site). All samples hybridized in the 2.2 kb range which is consistent with an *rn5s1*-eccrDNA monomer [12]. Cytoplasmic but not nuclear samples additionally hybridized at approximately 12 kb. Since our protocol includes an initial filtration step, higher molecular weight DNA could have been only extracted in a supercoiled or otherwise condensed state. The signal specificity indicates multicopy *rn5s1*eccDNA, possibly due to recombination-dependent concatemeric DNA replication as previously described for *Drosophila* [22]. Both, the resistance to full BamHI restriction and cytoplasm-specific location of higher-order *rn5s1*-eccrDNA need to be addressed by future studies.

To further investigate the observed resveratrol-induced cytoplasmic *rn5s1*-eccrDNA increase in HEK-293 cells, we sought to distinguish between nuclear import and cytoplasmic replication. We thus implemented a 12h treatment regimen involving combinations of resveratrol (50 μ M) and WGA (0.1mg/ml) to block nuclear transport, and control lectin ConA

(0.1 mg/ml) (Figure 3A and B). For resveratrol alone and in combination with ConA, we measured respective cytoplasmic *rn5s1*-eccrDNA increases of 180% and 100% ($P=0.01$) above control levels. WGA alone significantly reduced cytoplasmic *rn5s1*-eccrDNA to about 50%, whereas WGA plus resveratrol restored levels to 80% baseline. At the same time, resveratrol alone depleted nuclear *rn5s1*-eccrDNA significantly to about 30% ($P=0.01$) of the control baseline. Treatment with WGA or WGA plus resveratrol resulted in rescue tendencies toward baseline which were, however, not statistically significant. These results indicate transport of *rn5s1*-eccrDNA across the nuclear envelope but do not rule out potential compound tributaries through cytoplasmic *rn5s1*-eccrDNA amplification.

Furthermore, we captured 48 additional eccDNA molecules (average: 636 bp) without chromosomal prevalence and with only 1 duplication. While the majority of those eccDNAs were associated with non-repetitive genomic loci, three of them mapped to tandemly arranged gene clusters: *rn5S1* (98 repeat average), *pcdha1* (15 repeats) and *spanx4* (5 repeats) and another three were mitochondrial (Figure 4). Out of 45 non-mitochondrial sequences, 30 were intergenic, 12 mapped to intron/

Figure 3: Increases in cytoplasmic *rn5s1*-eccDNA involve nuclear transport in HEK-293 cells. QPCR analysis (relative to *alu*-eccDNA) of *rn5s1*-eccDNA under various conditions and relative to untreated controls (baseline). Chemical reagents used: Resveratrol (R); concavalin A (ConA; control lectin); wheat germ agglutinin (WGA; nuclear transport inhibitor). **A.** Nuclear extracts show a significant resveratrol-dependent reduction in *rn5s1*-eccDNA (-70%; R) which is WGA-sensitive (-20%; R&WGA). **B.** With resveratrol, cytoplasmic *rn5s1*-eccDNA surges (+180% R; +100% R&ConA) and returns to baseline with WGA (R&WGA). WGA by itself reduces baseline levels of *rn5s1*-eccDNA significantly (-50%). Values are means $\pm$ SEM.

* indicates statistical significance of annotated P; n = 12 per group.

regulatory region boundaries and 3 to coding exon/intron boundaries with further details summarized in Table 1.

Through further analysis with the repetitive DNA sequence mining tool CENSOR [16], we found that 33 of the 45 genomic sequences included 1-4 short repetitive DNA elements (averages 1.7). Of those, 91% were classified as transposable elements encompassing class-I retrotransposons [long interspersed nuclear elements (LINEs; includes *1*); short interspersed nuclear elements (SINEs; most abundant class in mammals; includes *aluJb*, *aluJo*, *aluSc*); endogenous retroviruses (ERV1 & 2)] and class-II non-autonomous DNA hAT superfamily transposons (Table 1). The abundance of these genomic elements is known to facilitate homologous recombination [23] and could therefore contribute to the mobilization of eccDNA. Three of the eccDNA clones that overlapped with actual coding exons did not include any such repetitive DNA elements and were therefore classified as recently discovered microDNAs [24]. While mechanisms are still at large,

our findings are in line with a paradigm based on DNA repetitiveness and mobilization. Our findings confirm the nature of previously described human eccDNA pools and validate our novel experimental approach.

In conclusion, we are introducing a novel and fast PCR-based approach facilitating the isolation, identification and relative quantification of extra-chromosomal circular DNA (eccDNA) obtained from cultured cells. The sensitivity and simplicity of this new methodology permit the study of the subcellular distribution and dynamics of specific eccDNA molecules in response to investigator-selected stimuli (i.e. pharmaceuticals) in a high-throughput fashion. The combination with standard recombinant DNA technology facilitates the identification of novel eccDNA molecules, such as the recently discovered class of coding microDNAs, under desired experimental conditions. This approach therefore provides a new set of tools to be used in the study of the physiology and pathology of eccDNA in general and eccrDNA in particular.

Figure 4: HEK-293 cells display genome-wide formation of eccDNA. This flowchart identifies the different types of eccDNA in HEK-293 cells and classifies short repetitive DNA elements found within. Our novel isolation and cloning strategy identified 48 eccDNA clones of which 13 map to coding genes (Table 1). The majority of the non-mitochondrial clones (31 with tandem repeats and 3 with unique sequences) contain 1-4 short repetitive DNA elements (with an average of 1.7) that are potentially responsible for their mobilization. The majority (91%) encode transposable elements with class-I retrotransposons (94%; LINE, SINE, *erv* and *alu* elements) being dominant over class-II DNA transposons (6%; *hAT*). The smaller fraction (9%) includes interspersed repeats such as *aluS* and *aluJ*.

Table 1: Identification and characterization of eccDNA linked to specific genes.

Gene	Chromosome	Element	Function	Size [bp]	Repetitive Element
acp6	1	int2	Mitochondrial Lipid Biosynthesis	598	aluSc (SINE); mer5A1 (hAT)
cdh9	5	int5	Promotes Cell-To-Cell Adhesion	650	l1hS (LINE); lipmA2 (LINE)
snx24	5	int3	Vesicular Membrane Transport	761	hervl (ERV)
aldh3a2	17	ex3-int3	Fatty Alcohol Oxidation	481	-
bicD1	12	int2	Cytoskeleton-Based RNA Sorting	781	aluS (IR); l1me1 (LINE)
cpE	4	int1	Neurotransmitter & Peptide Hormone Production	700	l1mb8 (LINE)
lacc1	13	int2	Leprosy & Crohn's Disease	560	aluJb (SINE)
nfyb	12	int6	Transcription Factor (Repressor)	460	aluJo (SINE)
parva	11	int1	Kidney Development; F-Actin Binding	672	-
pcdha1	5	int3	Neuronal Cell-Cell Interaction	870	-
plekhm3	2	int1	Intracellular Signal Transduction	437	aluJo (SINE)
rn5S1	1	5'utr	Ribosomal & Mitochondrial Function	661	hervip10fh (ERV)
sash1	6	int19-ex20	Tumor Suppression	629	-
specc1	17	int3	Structural Function	737	lipA8 (LINE)
spy1	7	int3	Cell-Cycle Regulation	708	aluJ(IR); aluJo (SINE)
tldr7	9	ex20-ex21	Lens Development	633	-

Overlapping exons (ex) and introns (int) are sequentially numbered and the upstream (5') position of the untranslated region (utr) in rn5S1 is indicated. Genes and repetitive elements are abbreviated according to standard convention. Other abbreviations: SINE: Short Interspersed Nuclear Elements; hAT: a class-II DNA transposon superfamily; LINE: Long Interspersed Nuclear Elements; ERV: Endogenous Retrovirus; IR: Interspersed Repeat

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Competing Interest Statement

The authors declare no competing interests.

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